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Research article

Metabolomics analysis of 'Housui' Japanese pear flower buds during endodormancy reveals metabolic suppression by thermal fluctuation

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ABSTRACT

Dormancy is a complex phenomenon that allows plants to survive the winter season. Studies of dormancy have recently attracted more attention due to the expansion of temperate fruit production in areas under mild winters and due to climate changes. This study aimed to identify and characterize the metabolic changes induced by chilling temperatures, as well as during thermal fluctuation conditions that simulate mild winter and/or climate change scenarios. To do this, we compared the metabolic profile of Japanese pear flower buds exposed to constant chilling at 6 °C and thermal fluctuations of 6 °C/18 °C (150 h/150 h) during endodormancy. We detected 91 metabolites by gas chromatography paired with time-of-flight mass spectrometry (GC-TOF-MS) that could be classified into eight groups: amino acids, amino acid derivatives, organic acids, sugars and polyols, fatty acids and sterols, phenol lipids, phenylpropanoids, and other compounds. Metabolomics analysis revealed that the level of several amino acids decreased during endodormancy. Sugar and polyol levels increased during endodormancy during constant chilling and might be associated with chilling stress tolerance and providing an energy supply for resuming growth. In contrast, thermal fluctuations produced low levels of metabolites related to the pentose phosphate pathway, energy production, and tricarboxylic acid (TCA) cycle in flower buds, which may be associated with failed endodormancy release. This metabolic profile contributes to our understanding of the biological mechanism of dormancy during chilling accumulation and clarifies the metabolic changes during mild winters and future climate change scenarios.

1. Introduction

Dormancy in plants is defined as a period of inhibited growth in meristems during favorable conditions (Rohde and Bhalerao, 2007), which allows perennial trees to survive cold winters and resume growth in the following spring (Saure, 1985). The induction and release of endodormancy is achieved by sufficient exposure to chilling conditions (Heide and Prestrud, 2005); the required amount of chilling to release endodormancy is controlled genetically and differs among genotypes (Egea et al., 2003). However, when chilling requirements are not met during winter, bud endodormancy release does not occur and leads to poor flowering in the spring (Sugiura and Honjo, 1997).

Japanese pear (*Pyrus pyrifolia* Nakai) is an important fruit produced in Japan and in the recent decades its production has expanded around the world; however, Japanese pear production is negatively affected in subtropical areas with mild winter conditions (Petri and Herter, 2002), where the chilling temperatures are not sufficient to release endodormancy. In mild winter areas, abnormal patterns of bud break and development has been observed in deciduous fruit trees due to the lack of chilling during the winter period (Erez and Couvillon, 1987; Viti et al., 2010). Thermal fluctuation that usually occurs in mild winters also has a negative effect on bud dormancy release (Anzanello et al., 2014). Furthermore, frequent warm winters due to global warming can affect most commercial cultivars due to insufficient chilling temperatures. Changes in winter temperatures will affect many major fruit production areas worldwide, and fruit growers will have difficulties with production in the future (Luedeling et al., 2011). The endodormancy release of fruit tree buds may not occur properly as climate

Abbreviations: CH, chilling hours; CU, chilling unit; GC-TOF-MS, gas chromatography paired with time-of-flight mass spectrometry; H_2O_2, hydrogen peroxide; PCA, principal component analysis; PC1, principal component 1; PC2, principal component 2; ROS, reactive oxygen species; TCA, tricarboxylic acid

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change or global warming progresses and affect new organ growth in the spring (Sugiura et al., 2007). In future climate change scenarios, it is expected that higher temperatures and frequent thermal fluctuation during winter months will negatively affect endodormancy release. Therefore, it is essential to study the effects of thermal fluctuation on endodormancy progression in order to understand and minimize problems related to dormancy release in mild winter conditions.

Several studies have focused on the different physiological aspects during endodormancy progression in attempt to uncover the mechanism of dormancy release. Bud dormancy release is associated with changes in the respiration rate and content of carbohydrates, polyamines, and organic acids that could affect bud break and flowering (Wang et al., 1987; Wang and Faust, 1994). Changes in amino acids have been reported during bud dormancy in grapes and apples (Ben Mohamed et al., 2010; Seif El-Yazal and Rady, 2012). In another study, changes in fatty acids and sterol were observed during bud break in apples (Wang and Faust, 1988). Carbohydrate metabolism during dormancy in the Japanese pear has been investigated (Ito et al., 2013; Hussain et al., 2015; Horikoshi et al., 2017). Carbohydrates serve as the main source of energy for metabolic changes and may control bud growth and development during dormancy and dormancy release (Sherson et al., 2003). However, insufficient chilling during dormancy may disturb carbohydrate dynamics (Bonhomme et al., 2005). In our previous report, we found that thermal fluctuation affected carbohydrate metabolism during endodormancy in Japanese pear flower buds (Horikoshi et al., 2017). Although several works have been done to understand the dormancy mechanism in deciduous trees, limited work has been conducted on the metabolic changes in endodormancy during thermal fluctuation conditions in Japanese pear.

Recently, transcriptome and proteome profiling have been conducted in perennial buds during dormancy (Horvath et al., 2008; Victor et al., 2010; Bi et al., 2011; Bai et al., 2013; Takemura et al., 2015; Porto et al., 2015). Transcriptomic analysis conducted in dormant Japanese pear buds indicated that genes related to stress responses, cell cycle, phytohormones, and water and energy are involved in endodormancy maintenance and in the transition from endodormancy to ecodormancy in Japanese pear (Nishitani et al., 2012; Liu et al., 2012; Bai et al., 2013; Takemura et al., 2015). The transcriptomic analysis of 'Suli' pear (*Pyrus pyrifolia* white pear group) revealed that several physiological processes such as phenylpropanoid biosynthesis, zeatin biosynthesis, ether lipid metabolism, glycerophospholipid metabolism, photosynthesis, phenylalanine metabolism, and starch and sucrose metabolism were all regulated differentially during bud dormancy (Liu et al., 2012). In grapes, the microarray analysis from buds under chilling accumulation showed that the majority of significantly differentially expressed genes were down-regulated (Mathiason et al., 2009). Although several works have reported interesting results in a genetic level during dormancy, metabolic studies are limited but necessary, as metabolites are the ultimate response to genetic or environmental changes (Fiehn, 2002).

The development of metabolomics techniques has improved our understanding of the complex molecular interactions of biological systems. However, there are only a few studies on metabolomics during dormancy in deciduous fruit trees, and to our knowledge metabolomics during bud endodormancy release in chilling and thermal fluctuation conditions has not yet been conducted in Japanese pear. Therefore, identifying and characterizing the metabolic profile of pear buds during endodormancy could confirm previous transcriptome results and provide essential information that will enrich our knowledge of the mechanism of dormancy release in Japanese pear. In this study, we compared the metabolic profiles of Japanese pear flower buds under either constant chilling conditions or fluctuating temperatures to identify metabolic changes induced by chilling temperatures during endodormancy, as well to characterize the metabolic changes that occur in conditions that simulate mild winter and/or climate change scenarios.

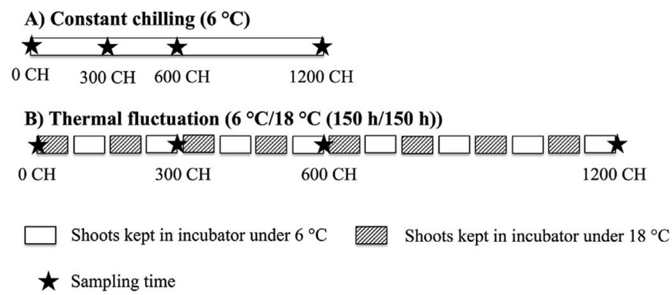

Fig. 1. Schematic of the experimental treatments for (A) shoots exposed to constant chilling at 6 °C during endodormancy, and (B) shoots exposed to thermal fluctuations of 6 °C/18 °C (150 h/150 h) during endodormancy.

2. Materials and methods

2.1. Plant materials and treatments

The experiment was conducted with one-year-old 'Housui' Japanese pear shoots that were approximately 90 cm in length and 1 cm in diameter, collected at the onset of chilling accumulation on October 28 and 29 2015 from trees grown in the Tsukuba-Plant Innovation Research Center (T-PIRC) of University of Tsukuba, Japan. The shoots were placed in bottles of distilled water and moved to incubators set at two different treatment conditions: A) a constant chilling condition of 6 °C and B) a thermal fluctuation condition of 6 °C/18 °C (150 h/150 h). Chilling hours (CH) were defined as the number of hours below 7.2 °C (Weinberger, 1950) and chilling unit (CU) values were calculated using the Saitama method (Asano and Okuno, 1990). Lateral flower buds were collected before treatment (0 CH) and after 300 CH, 600 CH, and 1200 CH in either constant chilling or thermal fluctuation treatments. Samples were frozen in liquid nitrogen and kept at −80 °C until further analysis. Shoot ends were cut off and water changed in 2 or 3 days intervals during the experiment period.

A schematic model of the experimental treatments is described in Fig. 1. The progress of chilling accumulation during endodormancy in constant chilling and thermal fluctuation conditions was measured in chilling hours (CH) (Weinberger, 1950). In shoots exposed to 300 CH, 600 CH, and 1200 CH under constant chilling at 6 °C, the CU values were 300, 600 and 1,200, respectively. Chilling progress calculated using the CU model for shoots exposed to thermal fluctuation of 6 °C/18 °C (150 h/150 h) during 300 CH, 600 CH, and 1200 CH were considered to be 150 CU, 300 CU, and 600 CU, respectively.

2.2. Determination of bud burst percentage and number of flower/cluster

The bud burst percentage in both treatments at each chilling accumulation time was calculated relative to the total number of lateral flower buds observed from five shoots. The five shoots from 0 CH, 300 CH, 600 CH, and 1200 CH under constant chilling and thermal fluctuation treatments were placed into a phytotron set at 25 ± 1 °C in a 16:8 h light:dark photoperiod. The bud burst was recorded in 2 days intervals and considered when buds were at the "mouse-ear" stage, or phenological stage C3, when the swelling of flower buds is apparent and green leaf tips are visible above the bud scales (Calvet and Guirbal, 1979; Coutanceau, 1971). The number of flowers per cluster was counted once the petals had completely opened flowers.

2.3. Metabolite analysis and profiling

The lateral floral buds from five shoots per sample (21 samples in total) were ground into a fine powder using a multi-bead shocker homogenizer (Yasui Kikai, Japan) and immediately shipped frozen to RIKEN (Japan) for metabolite profiling. Metabolite profiling and data processing details were provided according to the Metabolomics Society

Standard Initiative guidelines (Sansone et al., 2007). The equivalent of 5.6 mg fresh weight of each sample was injected into gas chromatography paired with time-of-flight mass spectrometry (GC-TOF-MS) (LECO, USA). The GC-TOF-MS analysis was performed as described by Kusano et al. (2011). Metabolite identification was conducted by referring to retention index and also by mass spectrometry fragmentation patterns with authentic standards. For normalization of the chromatograms, the cross-contribution compensating multiple standard normalization algorithm was used (Redestig et al., 2009).

2.4. Statistical analysis

The experimental design was completely randomized within the two treatment groups; there were four chilling accumulation time points (0 CH, 300 CH, 600 CH, and 1200 CH) that included three replicates. Metabolome data were normalized by a $\log_{10}$ transformation before being processed by principal component analysis (PCA). The significant fold changes of the 91 detected metabolites identified between treatments during endodormancy were calculated from three biological replicates and analyzed with an unpaired *t*-test test at 5% probability. All statistical analyses were performed using JMP statistical software (JMP, version 13.0; SAS Institute Inc., Cary, NC). The heat map was produced using GraphPad software (GraphPad Prism, version 7.0).

3. Results

3.1. Bud burst percentages and number of flowers/cluster

The bud burst (%) of flower buds exposed to the constant chilling treatment markedly increased along with chilling accumulation, reaching 100% at 1200 CH (Fig. 2A). Flower buds exposed to constant chilling exhibited a higher bud burst (%) than buds exposed to thermal fluctuation treatment at 1200 CH. The number of flowers per cluster was similar between these treatments at 300 CH, 600 CH, and 1200 CH (Fig. 2B). These results indicate that thermal fluctuation negatively affected bud burst (%) in Japanese flower buds.

3.2. PCA of metabolic changes during endodormancy under different temperature conditions

To investigate the metabolic changes during endodormancy of Japanese pear under different thermal conditions, we analyzed the metabolic profiles of lateral flower buds exposed to either constant chilling or thermal fluctuation treatments. We identified and quantified 91 metabolites and analyzed the distribution of these metabolites using PCA (Fig. 3). The PCA analysis showed that 50.2% of the variance in metabolites was explained by the first two principal components, PC1 (27.4%) and PC2 (22.8%). The PCA plots were able to differentiate between thermal conditions and accumulated CH, suggesting that some of the metabolites changed between treatments and along the chilling accumulation. Although PC1 clearly distinguished 0 CH from flower buds that were exposed to constant chilling or thermal fluctuation

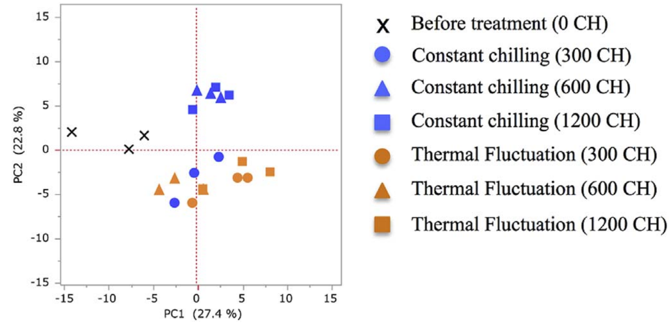

Fig. 3. Principal component analysis (PCA) score plots of 91 metabolites from three biological replicates of 'Housui' Japanese pear flower buds collected before (0 CH) and after being exposed to constant chilling at 6 °C and thermal fluctuation of 6 °C/18 °C (150 h/150 h) for 300 CH, 600 CH, and 1200 CH. Scores are plotted with the first principal component (PC1) represented in the x-axis and the second principal component (PC2) in the y-axis. The variance explained by each principal component is shown in parentheses.

treatments during endodormancy, it could not distinguish between treatments. However, PC2 clearly separated the plots of buds exposed to a constant chilling treatment at 600 CH and 1200 CH where it there were the highest percentages of bud burst, from buds that were exposed to a constant chilling at 300 CH and thermal fluctuation treatment at 300 CH, 600 CH, and 1200 CH that had the lowest percentage of bud burst after treatment exposure. This result suggests that some metabolites changed in the constant chilling treatment and led to high percentages of bud burst; however, at 300 CH in the constant chilling and thermal fluctuation treatments, the changes in metabolites were insufficient to induce bud burst and led to lower bud burst percentages. The PC2 loading values were investigated to identify which metabolites had an influence on PC2 (Supplementary Fig. 2). Ribitol, *trans*-3-caffeoyl-quinate, (+)-catechin hydrate, lactitol, and lactose had the highest positive PC2 loading values. In contrast, 3-cyanoalanine, pipecolate, and ornithine had the highest negative PC2 loading values. For PC1, raffinose, galactinol, and itaconate had the highest positive loading values, while serine, threonine, and valine had the highest negative values (Supplementary Fig. 1).

3.3. Changes on metabolite levels under different temperature conditions

To identify the metabolic changes during endodormancy in different temperature conditions, we analyzed the responses of 91 detected metabolites under constant chilling and thermal fluctuation treatments by computing the fold-change relative to 0 CH samples (Fig. 4). The grouping of metabolites was done based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://kegg.jp/) and Human Metabolome Database (HMDB) (http://www.hmdb.ca). Metabolites were divided into eight groups: amino acids (21), amino acids derivatives (4), organic acids (25), sugar and polyols (16), fatty acids and sterols (5), phenol lipids (6), phenylpropanoids (3), and other compounds (11). We observed that many of the detected metabolites changed during

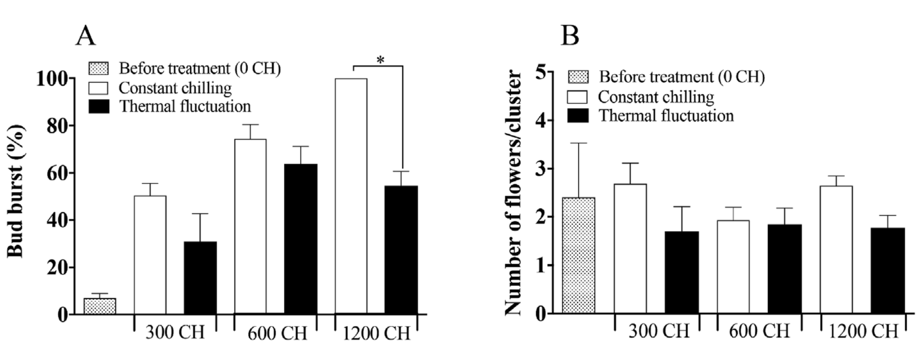

Fig. 2. (A) Bud burst (%), and (B) number of flowers/cluster in 'Housui' Japanese pear flower buds before treatment (0 CH) and after being exposed to constant chilling at 6 °C and thermal fluctuations of 6 °C/18 °C (150 h/150 h) for 300 CH, 600 CH, and 1200 CH during endodormancy. Shoots were maintained in a phytotron set at 25 ± 1 °C and a 16:8 h light: dark photoperiod. Asterisks (*) indicate significant differences between treatments at 300 CH, 600 CH, or 1200 CH determined by an unpaired *t*-test at $p < 0.05$.

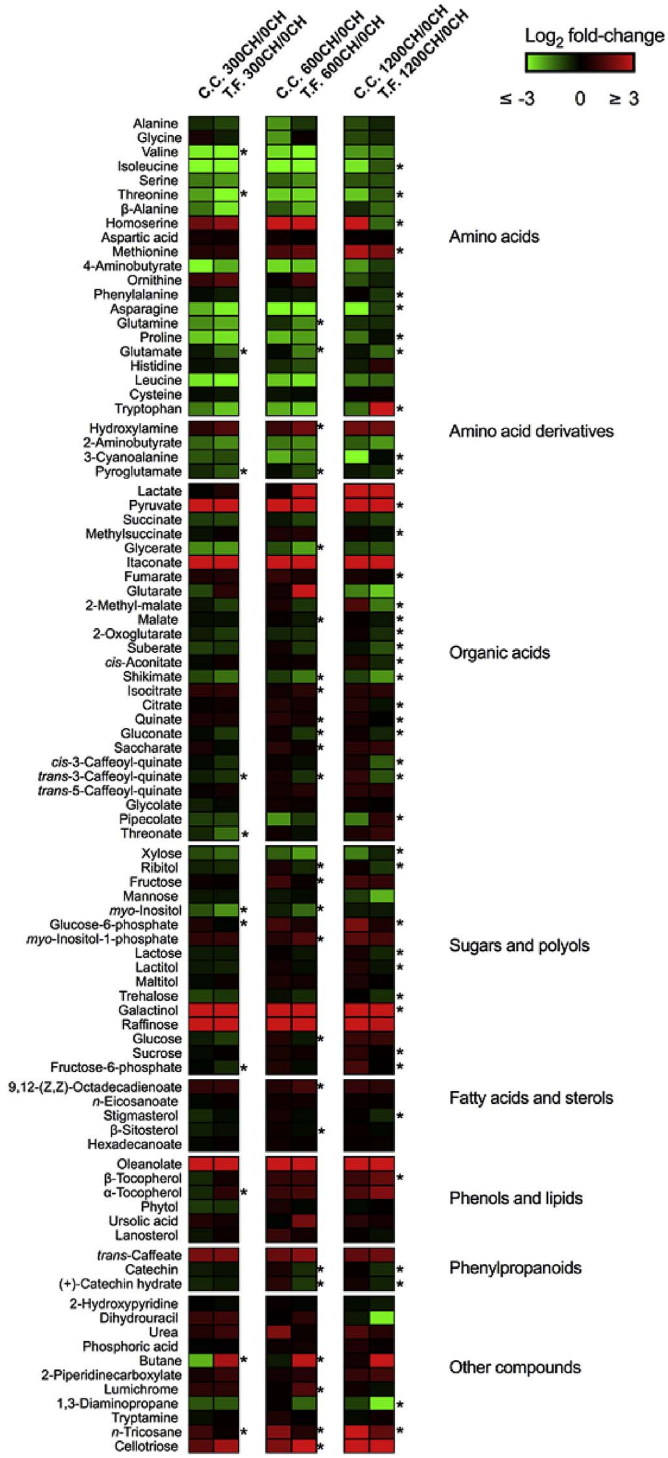

Fig. 4. Heat map of fold-changes relative to 0 CH for 91 detected metabolites in flower buds exposed to constant chilling at 6 °C, which are represented by the letters C.C., and thermal fluctuation conditions of 6 °C/18 °C (150 h/150 h) are represented by the letters T.F. for 300 CH, 600 CH, and 1200 CH. Values represent the means of $\log_2$-transformed fold-changes from three biological replicates relative to the 0 CH (before treatment). Asterisks (*) indicate significant differences between treatments at 300 CH, 600 CH, or 1200 CH determined by an unpaired *t*-test at $p < 0.05$.

endodormancy when exposed to different temperature conditions. The level of most amino acids tended to decrease regardless to the treatment exposure during endodormancy in Japanese pear flower buds. Proline levels tended to decrease at the beginning of endodormancy regardless to the treatment, while methionine tended to increase. The level of glutamate and pyroglutamate were lower in flower buds exposed to thermal fluctuation than constant chilling treatments. The level of organic acids such as pyruvate tended to increase during endodormancy. The level of malate was lower in flower buds exposed to thermal fluctuation treatment at 600 CH and 1200 CH. Furthermore, citrate and 2-oxoglutarate levels were lower in flower buds exposed to thermal fluctuation treatment at 1200 CH. Similarly, *trans*-3-caffeoyl-quinate exhibited lower levels in the thermal fluctuation treatments. Most of the sugars and polyols tended to increase in the constant chilling treatment during endodormancy. Levels of galactinol and raffinose tended to increase in flower buds in both treatment groups. The levels of sucrose, lactose, and trehalose were lower in flower buds exposed to thermal fluctuation compared to the constant chilling treatment at 1200 CH. The fructose and glucose levels were lower in buds exposed to thermal fluctuation treatment at 600 CH. Similarly, glucose-6-phosphate and fructose-6-phosphate levels were lower in buds exposed to thermal fluctuation treatment at 300 CH and 1200 CH. In addition, the level of lactitol was lower in buds exposed to the thermal fluctuation treatment at 1200 CH. In the fatty acid and sterols group, β-sitosterol and stigmasterol levels were lower in buds exposed to thermal fluctuation treatments at 600 CH and 1200 CH, respectively. Both phenol lipids α-tocopherol and β-tocopherol tended to increase in buds from both treatments during endodormancy. The level of flavonoid catechin was lower in the thermal fluctuation treatment compared to the constant chilling treatment at 600 CH and 1200 CH. These results indicate that several metabolites were suppressed during endodormancy progression under thermal fluctuation.

3.4. Global view of metabolic changes affected by different temperature conditions

The significant metabolite changes in buds exposed to constant chilling compared to thermal fluctuation treatments throughout the whole metabolic cycle is shown in Fig. 5. Most metabolites involved in “pentose and glucuronate interconversions” and “starch and sucrose metabolism” pathways were inhibited in buds exposed to thermal fluctuation during endodormancy. The metabolic pathway involved in the biosynthesis of chlorogenic acid was also induced in flower buds under constant chilling. However, valine, leucine, and isoleucine biosynthesis was inhibited during endodormancy in both constant chilling and thermal fluctuation treatments. Furthermore, glutamate and pyroglutamate biosynthesis were inhibited during endodormancy in buds exposed to thermal fluctuation treatment.

4. Discussion

In this study, we investigated the effect of constant chilling at 6 °C and thermal fluctuations of 6 °C/18 °C (150 h/150 h) on the metabolic functions in 'Housui' Japanese pear lateral flower buds. We adopted 6 °C as an effective temperature in our experimental conditions, which was based on previous reports that showed that a range between 0 and 6 °C was effective in releasing endodormancy in Japanese pear (Sugiura and Honjo, 1997). We also exposed flower buds to fluctuating temperatures of 6 °C/18 °C (150 h/150 h) in an attempt to simulate mild winter conditions that are usually followed by thermal fluctuation of alternating chilling and non-chilling temperatures. We have adopted 18 °C as the non-chilling temperature based on the report of Sugiura and Honjo (1997), which indicated that temperatures higher than 12 °C do not release endodormancy in Japanese pear, but 18 °C can have negative effects on endodormancy release (Asano and Okuno, 1990).

The bud burst percentages gradually increased in flower buds during chilling accumulation increased during endodormancy in constant chilling treatments (Fig. 2A). Sugiura and Honjo (1997) reported that 750 h in a temperature range of 0–6 °C was needed to release endodormancy in 'Kousui' Japanese pear. In our hands, bud burst reached 74.5% and 100% when buds were exposed to constant chilling treatments for 600 CH and 1200 CH, respectively. Endodormancy release in

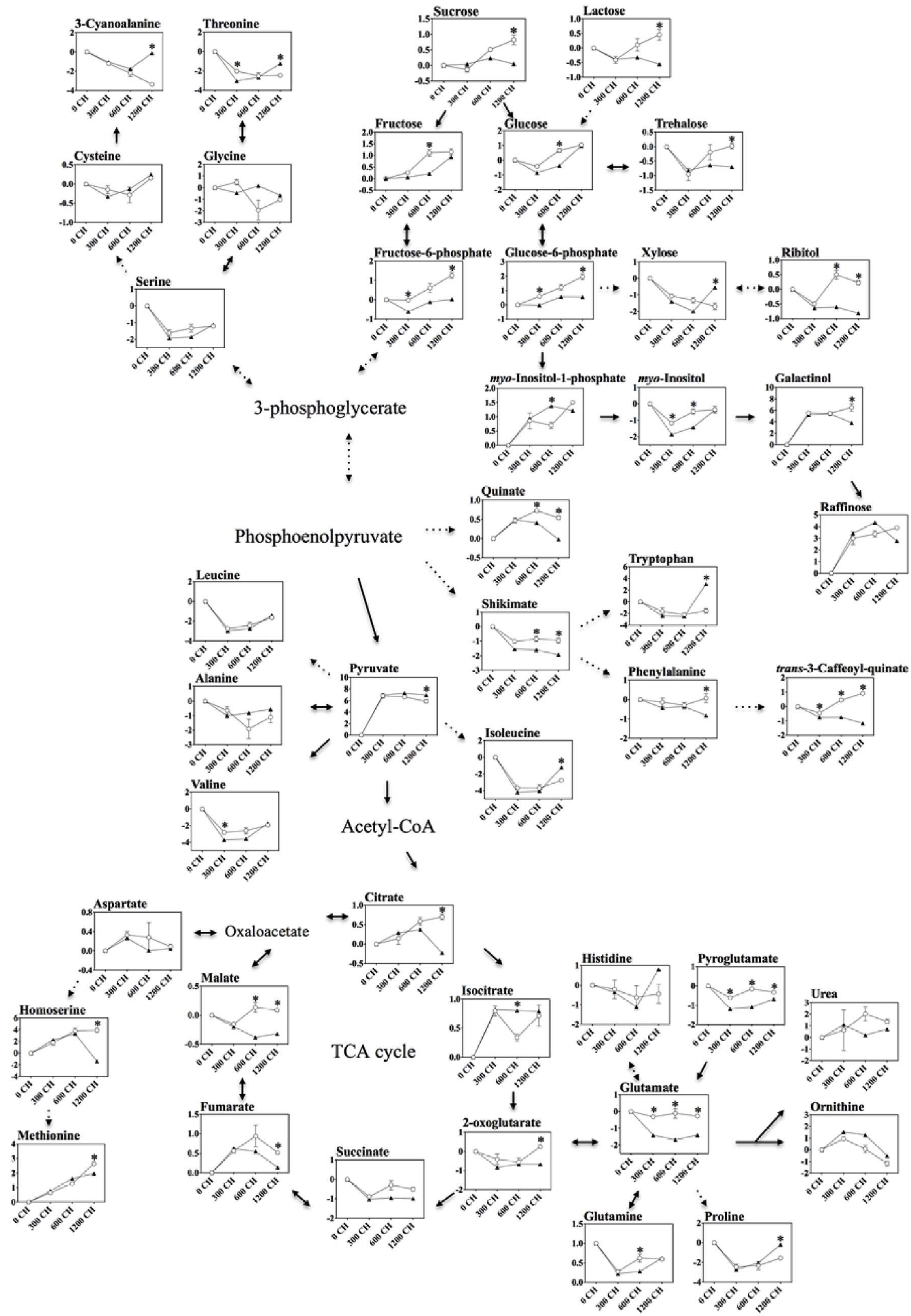

Fig. 5. Changes of metabolite levels in metabolic pathways in 'Housui' Japanese pear flower buds collected at 0 CH and exposed to constant chilling at 6 °C (open circles) and thermal fluctuations of 6 °C/18 °C (150 h/150 h) (close triangles) for 300 CH, 600 CH, and 1200 CH. Values represent the means of $\log_2$-transformed fold-changes from three biological replicates relative to 0 CH. Asterisks (*) indicate significant differences between treatments at 300 CH, 600 CH, or 1200 CH determined with an unpaired *t*-test at $p < 0.05$.

Japanese pear occurs when the bud break percentage reaches more than 70% (Takemura et al., 2013). Thus, dormancy release was considered occur in flower buds exposed to constant chilling at 6 °C after 600 CH and 1200 CH. In contrast, the bud burst percentages in buds exposed to thermal fluctuation treatments reached maximum values of 63.9% that then decreased after 1200 CH. These results indicate that thermal fluctuation treatments negatively affected the fulfillment of chilling requirements in Japanese pear flower buds. We also obtained similar results in our previous report, where thermal fluctuations had a negative impact on bud burst in Japanese pear flower buds (Horikoshi et al., 2017). The decrease of bud burst percentages at 1200 CH to 54.7% suggests that long periods of exposure to thermal fluctuation treatments negatively affect endodormancy. In the thermal fluctuation treatment reached a maximum accumulation of 600 CU at 1200 CH of exposure. The non-chilling temperature, which is considered an ineffective temperature to release endodormancy (Asano and Okuno, 1990), negatively affected endodormancy release and CU units in the thermal fluctuation conditions. Thus, these results indicate that in areas with mild winters with periods of non-chilling temperatures, those temperatures may be a crucial factor for achieving dormancy release. Although it has been reported that higher temperatures during winter caused altered flower bud development in apricots (Viti et al., 2010), the number of flowers/cluster was not affected by the temperature conditions in our study (Fig. 2B).

We observed that the level of most amino acids decreased in flower buds during endodormancy regardless to the treatment they received (Fig. 4). When carbohydrate supply is limited, the demand for energy can be provided by amino acid oxidation and the degradation of other storage compounds (Galili et al., 2014). In general, the amino acids produced by protein degradation can be converted to pyruvate or acetyl-CoA before entering the tricarboxylic acid (TCA) cycle or they can be converted directly into one of the TCA cycle intermediates, such 2-oxoglutarate (Araújo et al., 2011). Our results indicate that pyruvate levels increased during endodormancy in both temperature treatments, suggesting that some amino acids were possibly catabolized to pyruvate during endodormancy in Japanese pear flower buds to serve as an energy source. During endodormancy, leucine, isoleucine, and valine decreased regardless to the treatment. Thus, we suggest that amino acid catabolism may be essential for endodormancy in Japanese pear flower buds and could contribute with the energy state during this period.

Proline levels decreased during endodormancy regardless to the treatment (Fig. 4). It has been reported in apple buds that seasonal changes in arginine and proline may play an important role in conferring chilling tolerance during winter (Seif El-Yazal et al., 2014). However, our results are consistent with those from Odlum et al. (1993) and Angelcheva et al. (2014), who reported that there is no correlation between cold acclimation or low temperature stress tolerance and changes in proline content in spruce needles. Thus, these results reveal that the chilling requirement is fulfilled in Japanese pear flower buds in constant chilling conditions of 6 °C, and that proline metabolism may not be involved in the development of chilling stress tolerance. Glutamate and pyroglutamate were inhibited in plants exposed to the thermal fluctuation treatment (Fig. 4). It has been reported in *Arabidopsis* that 5-oxoproline, also known as pyroglutamate, can be synthesized from glutathione and then converted to glutamate (Ohkama-Ohtsu et al., 2008). Glutathione is known to play a role during endodormancy release (Fuchigami and Nee, 1987), and can increase during chilling accumulation in Japanese pear flower buds (Zanol et al., 2010). Thus, thermal fluctuation treatment may inhibit the glutathione biosynthesis that led to low bud burst percentages and low glutamate and pyroglutamate levels in flower buds; however, further investigation is needed to test this hypothesis.

The methionine level increased in buds subjected to constant chilling treatment during the late endodormancy stage (Fig. 4). Methionine is an essential amino acid and the precursor to several metabolites including S-adenosyl methionine, which is the precursor of polyamines and ethylene (Amir et al., 2002). Recently, it has been reported that the ethylene response pathway plays a key role in dormancy release (Ophir et al., 2009). Methionine is also the precursor substrate of spermidine, and the expression of spermidine synthase increased in grape buds during chilling requirement fulfillment (Mathiason et al., 2009). Wang and Faust (1994) also reported that spermidine content significantly increased in apple flower buds during winter chilling accumulation. Although we did not analyze ethylene and polyamine levels, the synthesis of methionine in the buds during the late stage of endodormancy may still play an essential role in endodormancy release.

Most sugars and polyols exhibited high abundance in the constant chilling treatment during the late period of endodormancy (600 and 1200 CH) (Fig. 4). Sucrose levels were higher in buds exposed to constant chilling than thermal fluctuation treatments at 1200 CH. Sucrose provides energy for growth in the spring and may be important for conferring freezing tolerance in the winter (Yoshioka et al., 1988). An increase in sucrose-phosphate synthase activity leads to sucrose synthesis and is often observed in low temperature conditions (Ensminger et al., 2006). We observed higher levels of glucose and fructose in constant chilling compared to thermal fluctuation treatments at 600 CH. During bud break, the presence of glucose and fructose is related to bud growth capacity in peaches (Maurel et al., 2004). Furthermore, Hussain et al. (2015) reported that low glucose and fructose concentrations are associated with low bud break percentage in Japanese pear. Thus, low levels of sugars and polyols in buds in thermal fluctuation treatments during late endodormancy not only may impair protection against chilling stress, but also fail to provide sufficient energy for growth resumption in the spring.

Both raffinose and galactinol levels were higher in buds in both treatment groups (Fig. 4). Temperate plants acclimate to survive winter by synthesizing cryoprotective molecules such as raffinose, trehalose, ribitol, and inositol (Janská et al., 2010). A significant amount of raffinose accumulated in vegetative tissues of cold acclimated *Arabidopsis* plants (Zuther et al., 2004; Taji et al., 2002). Moreover, the levels of galactinol increased during the onset of low temperature tolerance in Siberian spruce (Angelcheva et al., 2014). Our findings confirm the results from Liu et al. (2012), in which they found that galactinol synthase was up-regulated during endodormancy periods in 'Suli' pear flower buds under natural chilling conditions. Raffinose biosynthesis requires sucrose and galactinol as precursors and raffinose synthase as the major enzyme (Taji et al., 2002). Thus, an abundant concentration of galactinol led to the increase in raffinose content in flower buds. Galactinol and raffinose may function as reactive oxygen species (ROS) scavengers that protect plant cells from oxidative damage and regulate redox homeostasis caused by stressful conditions (Nishizawa et al., 2008a, 2008b). Therefore, an increase in galactinol and raffinose levels in chilling conditions is strongly associated with the response against chilling stress that protects the flower buds from cold temperature damage and also may be involved in the control of dormancy through the regulation of ROS levels.

The levels of most metabolites involved in the TCA cycle such as citrate, 2-oxoglutarate, fumarate, and malate were higher in buds exposed to constant chilling than when exposed to thermal fluctuation treatment during endodormancy (Fig. 4). Tan et al. (2010) found that the TCA cycle was activated upon the release of dormancy in nectarine flower buds. Furthermore, Gai et al. (2013) found a slight up-regulation of 2-oxoglutarate dehydrogenase, succinate dehydrogenase, malate, and NAD-malate dehydrogenase in tree peonies as bud break began. The previous authors also suggested that pathways involved in carbohydrate metabolism and ATP production such as glycolysis, TCA cycle, pentose phosphate pathway, and oxidative phosphorylation were stimulated at the end of endodormancy to produce energy for bud sprouting. Although the levels of most metabolites involved in the TCA cycle did not markedly increase during endodormancy, their levels can be inhibited in buds exposed to thermal fluctuations. These results suggest that thermal fluctuation negatively affect metabolites related to

the TCA cycle during endodormancy and also may contribute to lower bud break percentages, as we observed in our experimental conditions.

Flower buds exposed to constant chilling treatment during endodormancy exhibited increased levels of glucose-6-phosphate and fructose-6-phosphate (Fig. 4). In grapes, bud dormancy release is associated with the activation of the pentose phosphate pathway (Halaly et al., 2008). Thus, considering the high levels of glucose-6-phosphate availability, it is possible that pentose phosphate pathway also plays a role during endodormancy in Japanese pear buds. However, other works reported that chilling deficiency maintained dormant nectarine floral buds in a state of increased pentose phosphate pathway activity (Tan et al., 2010). On the other hand, it is important to emphasize that H_2O_2 plays a role in endodormancy release in Japanese pear by initiating a cascade of reactions (Kuroda et al., 2002), and that H_2O_2 overflow can lead to the activation of glucose-6-phosphate dehydrogenase, which initiates the pentose phosphate pathway (Hu and Couvillon, 1990). The pentose phosphate pathway is also a way of recharging the antioxidant stress system with reduced NADPH, which is essential to induce ascorbate glutathione cycle (Salvemini et al., 1999; Yu et al., 2004). Gai et al. (2013) suggested that ascorbate-glutathione cycle was induced to control ROS, which are regulatory factors for bud break in tree peony. Thus, the pentose phosphate pathway is important for controlling dormancy by regulating ROS in Japanese pear flower buds, however when buds are exposed to thermal fluctuations, this pathway may not work properly and lead to a failure of endodormancy release.

In this study, the level of *trans*-3-caffeoyl-quinate (chlorogenic acid) increased during endodormancy in lateral flower buds exposed to constant chilling treatment (Fig. 4). Due to limited research, chlorogenic acid has not yet been considered a target metabolite involved in the dormancy. Chlorogenic acid is a common derivative of quinate, which has been reported to accumulate in the root tissue of many plant species (Cole, 1984; McClure, 1960; Maruta et al., 1995). Furthermore, chlorogenic acid may play a role in stress responses by acting as a potent hydrogen-donating antioxidant in plants (Grace and Logan, 2000). Wang et al. (1991) suggested that phenolic compounds such as chlorogenic acid might influence growth processes in dormant apple buds. In our study, chlorogenic acid levels increased during endodormancy in constant chilling, but not in the thermal fluctuation treatment. Thus, we suggest that this metabolite is involved in chilling stress tolerance and/or dormancy release; however, further studies are needed to characterize its importance for dormancy regulation mechanisms.

Transcript profiling has been conducted in an attempt to identify genes involved in chilling requirement fulfillment in grapes (Mathiason et al., 2009) and in Japanese pear to identify genes involved in the transition from endodormancy to ecodormancy (Nishitani et al., 2012; Liu et al., 2012; Bai et al., 2013; Takemura et al., 2015). Consistent with these works, we have also tried to identify potential metabolites involved in endodormancy regulation and release in Japanese pear. For example, lactitol and lactose levels increased during chilling fulfillment in constant chilling treatments and exhibited highly positive PC2 loading values (Supplementary Fig. 2). It has been reported that levels of lactose decrease in apricot flower buds during the onset of bud burst after the application of gibberellic acid (Zhuang et al., 2015); however, its function during endodormancy is poorly understood and further investigation is needed to clarify the involvement of these metabolites in the regulation of endodormancy in Japanese pear flower buds.

In this study, we investigated the metabolic changes that occur during endodormancy in lateral floral buds exposed to constant chilling or thermal fluctuation. We found that the metabolic profiles during endodormancy in buds exposed to thermal fluctuation clearly differed from buds exposed to constant chilling, with the greatest changes observed in sugar and polyol levels. The low levels of some sugars such as glucose, fructose, glucose-6-phosphate, and fructose-6-phosphate measured in buds exposed to thermal fluctuation during endodormancy may be associated with the low bud burst rates observed in this study. In contrast, the high levels of glucose-6-phosphate in buds exposed to constant chilling suggest that the pentose phosphate pathway plays a role in endodormancy release in Japanese pear buds. Finally, this work contributes to previously published results in efforts to fully elucidate the biological mechanism behind chilling accumulation during dormancy, which will allow for the development of new cultivars of deciduous fruit species that can adapt to the lower chilling conditions of warm winters and for future scenarios caused by climate change. Understanding the mechanism of dormancy initiation and release is a prerequisite for the development of more effective cultivation strategies and genetic control of dormancy release.

Contributions

Humberto Mitio Horikoshi and Sumiko Sugaya designed the experiment; Humberto Mitio Horikoshi performed the research; Makoto Kobayashi, Kazuki Saito and Miyako Kusano performed the metabolite analysis; Humberto Mitio Horikoshi and Sumiko Sugaya analyzed the data and wrote the paper; Yoshihiko Sekozawa helped to revise the paper.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2018.02.028.

References

Amir, R., Hacham, Y., Galili, G., 2002. Cystathionine γ-synthase and threonine synthase operate in concert to regulate carbon flow towards methionine in plants. Trends Plant Sci. 7, 153–156.
Angelcheva, L., Mishra, Y., Antti, H., Kjellsen, T.D., Funk, C., Strimbeck, R.G., Schröder, W.P., 2014. Metabolic analysis of extreme freezing tolerance in Siberian spruce (*Picea obovate*). New Phytol. 204, 545–555.
Anzanello, R., Fialho, F.B., dos Santos, H.P., Bergamaschi, H., Marodin, G.A.B., 2014. Bud dormancy in apple trees after thermal fluctuations. Pesq. Agropec. Brasil 49, 457–464.
Araújo, W.L., Tohge, T., Ishizaki, K., Leaver, C.J., Fernie, A.R., 2011. Protein degradation- an alternative respiratory substrate for stressed plants. Trends Plant Sci. 16, 489–498.
Asano, S., Okuno, T., 1990. Period of breaking the rest and the quality of chilling requirements of 'Kousui' and 'Housui' Japanese pear. Bull. Saitama Hort. Exp. Stn. 17, 41–47 (In Japanese with English abstract).
Bai, S., Saito, T., Sakamoto, D., Ito, A., Fujii, H., Moriguchi, T., 2013. Transcriptome analysis of Japanese pear (*Pyrus pyrifolia* Nakai) flower buds transitioning through endodormancy. Plant Cell Physiol. 54, 1132–1151.
Ben Mohamed, H., Vadel, M.A., Geuns, J.M.C., Khemira, H., 2010. Biochemical changes in dormant grapevine shoot tissues in response to chilling: possible role in dormancy release. Sci. Hortic. 124, 440–447.
Bi, Y.D., Wei, Z.G., Shen, Z., Lu, T.C., Cheng, Y.X., Wang, B.C., Yang, C.P., 2011. Comparative temporal analyses of the *Pinus sylvestris* L. var. *mongolica litv.* apical bud proteome from dormancy to growth. Mol. Biol. Rep. 38, 721–729.
Bonhomme, M., Rageau, R., Lacointe, A., Gendraud, M., 2005. Influences of cold deprivation during dormancy on carbohydrate contents of vegetative and floral primordia and nearby structures of peach buds (*Prunus persica* L. Batch). Sci. Hortic. 105, 223–240.
Calvet, C., Guirbal, M., 1979. Manual d'arboriculture fruitère. In: EPAGRI. 2001. J. B. Baillière, Paris, pp. 167 Nashi, a pera japonesa. Florianópolis: Epagri/JICA, 341 pp.
Cole, R.A., 1984. Phenolic acids associated with the resistance of lettuce cultivars to the lettuce root aphid. Ann. Appl. Biol. 105, 129–145.
Coutanceau, M., 1971. Fruticultura técnica y economía de lós cultivos de rosáceas

Leñosas productoras de fruta. In: EPAGRI. 2001. Oikostau, Barcelona, pp. 608 Nashi, a pera japonesa. Florianópolis: Epagri/JICA, 341 pp.
Egea, J., Ortega, E., Martynez-Gomez, P., Dicenta, F., 2003. Chilling and heat requirements of almond cultivars for flowering. Environ. Exp. Bot. 50, 79–85.
Ensminger, I., Busch, F., Hüner, N.P.A., 2006. Photostasis and cold acclimation: sensing low temperature through photosynthesis. Physiol. Plantarum 126, 28–44.
Erez, A., Couvillon, G.A., 1987. Characterization of the influence of moderate temperature on rest completion in peach. J. Am. Soc. Hortic. Sci. 112, 677–680.
Fiehn, O., 2002. Metabolomics-the link between genotypes and phenotypes. Plant Mol. Biol. 48, 155–171.
Fuchigami, L.H., Nee, C.C., 1987. Degree growth stage model and rest-breaking mechanisms in temperate woody perennials. Hortscience 22, 836–844.
Gai, S., Zhang, Y., Liu, C., Zhang, Y., Zheng, G., 2013. Transcript profiling of *Paoenia ostii* during artificial chilling induced dormancy release identifies activation of GA pathway and carbohydrate metabolism. PLoS One 8http://dx.doi.org/10.1371/journal.pone.0055297. e55297.
Galili, G., Avin-Wittenberg, T., Angelovici, R., Fernie, A.R., 2014. The role of photosynthesis and amino acid metabolism in the energy status during seed development. Front. Plant Sci. 5, 447.
Grace, S.C., Logan, B.A., 2000. Energy dissipation and radical scavenging by the plant phenylpropanoid pathway. Phil. Trans. Roy. Soc. Lond. B 355, 1499–1510.
Halaly, T., Pang, X., Batlkoff, T., Crane, O., Keren, A., Venkateswari, J., Ogrodovitch, A., Sadka, A., Lavee, S., Or, E., 2008. Similar mechanisms might be triggered by alternative external stimuli that induce dormancy release in grape buds. Planta 228, 79–88.
Heide, O.M., Prestrud, A.K., 2005. Low temperature, but not photoperiod, controls growth cessation and dormancy induction and release in apple and pear. Tree Physiol. 25, 109–114.
Horikoshi, M.H., Sekozawa, Y., Sugaya, S., 2017. Inhibition of carbohydrate metabolism by thermal fluctuations during endodormancy lead to negative impacts on bud burst and incidence of floral necrosis in 'Housui' Japanese pear flower buds. Sci. Hortic. 224, 324–331.
Horvath, D.P., Chao, W.S., Suttle, J.C., Thimmapuram, J., Anderson, J.V., 2008. Transcriptome analysis identifies novel responses and potential regulatory genes involved in seasonal dormancy transitions of leafy spurge (*Euphorbia esula* L.). BMC Genom. 9, 536.
Hu, H., Couvillon, G.A., 1990. Activities of catalase and pentose phosphate pathway dehydrogenase during dormancy release of nectarine seed. J. Am. Soc. Hortic. Sci. 115, 987–990.
Hussain, S., Niu, Q., Yang, F., Hussain, N., Teng, Y., 2015. Possible role of chilling in floral and vegetative bud dormancy release in *Pyrus pyrifolia*. Biol. Plant. 59, 726–734.
Ito, A., Sugiura, T., Sakamoto, D., Moriguchi, T., 2013. Effects of dormancy progression and low-temperature response on changes in the sorbitol concentration in xylem sap of Japanese pear during winter season. Tree Physiol. 33, 398–408.
Janská, A., Marsik, P., Zelenkova, S., Ovesna, J., 2010. Cold stress and acclimation-what is important for metabolic adjustment? Plant Biol. 12, 395–405.
Kuroda, H., Sugiura, T., Ito, D., 2002. Changes in hydrogen peroxide content in flower buds of Japanese pear (*Pyrus pyrifolia* Nakai) in relation to breaking of endodormancy. J. Jpn. Soc. Hortic. Sci. 71, 610–616.
Kusano, M., Redestig, H., Hirai, T., Oikawa, A., Matsuda, F., Fukushima, A., Watanabe, S., Yano, M., Hiwasa-Tanase, K., Ezura, H., Saito, K., 2011. Covering chemical diversity of genetically-modified tomatoes using metabolomics for objective substantial equivalence assessment. PLoS One 6http://dx.doi.org/10.1371/journal.pone.0016989. e16989.
Liu, G., Li, W., Zheng, P., Xu, T., Chen, L., Liu, D., Hussain, S., Yuanwen, T., 2012. Transcriptomic analysis of 'Suli' pear (*Pyrus pyrifolia* white pear group) buds during the dormancy by RNA-Seq. BMC Genom. 13, 700.
Luedeling, E., Girvetz, E.H., Semenov, M.A., Brown, P.H., 2011. Climate change affects winter chill for temperate fruit and nut trees. PLoS One 6http://dx.doi.org/10.1371/journal.pone.0020155. e20155.
Maruta, Y., Kawabata, J., Niki, R., 1995. Antioxidative caffeoylquinic acid derivatives in the roots of burdock (*Arctium lappa* L.). J. Agric. Food Chem. 43, 2592–2595.
Mathiason, K., He, D., Grimplet, J., Venkateswari, J., Galbraith, D.W., Or, E., Fennell, A., 2009. Transcript profiling in *Vitis riparia* during chilling requirement fulfillment reveals coordination of gene expression patterns with optimized bud break. Funct. Integr. Genom. 9, 81–96.
Maurel, K., Leite, G.B., Bonhomme, M., Guilliot, A., Rageau, R., Pétel, G., Sakr, S., 2004. Trophic control of bud break in peach (*Prunus persica*) trees: a possible role of hexoses. Tree Physiol. 24, 579–588.
McClure, T.T., 1960. Chlorogenic acid accumulation and wound healing in sweet potato roots. Am. J. Bot. 47, 277–280.
Nishitani, C., Saito, T., Ubi, B.E., Shimizu, T., Itai, A., Saito, T., Yamamoto, T., Moriguchi, T., 2012. Transcriptome analysis of *Pyrus pyrifolia* leaf buds during transition from endodormancy to ecodormancy. Sci. Hortic. 147, 49–55.
Nishizawa, A., Yabuta, Y., Shigeoka, S., 2008a. The contribution of carbohydrates including raffinose family oligosaccharides and sugar alcohols to protection of plant cells from oxidative damage. Plant Signal. Behav. 11, 1016–1018.
Nishizawa, A., Yabuta, Y., Shigeoka, S., 2008b. Galactinol and raffinose constitute a novel function to protect plants from oxidative damage. Plant Physiol. 147, 1251–1263.
Odlum, K., Blake, T., Kim, Y., Glerum, C., 1993. Influence of photoperiod and temperature on frost hardiness and free amino acid concentrations in black spruce seedlings. Tree Physiol. 13, 275–282.
Ohkama-Ohtsu, N., Oikawa, A., Zhao, P., Xiang, C., Saito, K., Oliver, D.J., 2008. A gamma-glutamyl transpeptidase-independent pathway of glutathione catabolism to glutamate via 5-oxoproline in Arabidopsis. Plant Physiol. 148, 1603–1613.
Ophir, R., Pang, X., Halaly, T., Venkateswari, J., Lavee, S., Galbraith, D., Or, E., 2009. Gene-expression profiling of grape bud response to two alternative dormancy-release stimuli expose possible links between impaired mitochondrial activity, hypoxia, ethylene-ABA interplay and cell enlargement. Plant Mol. Biol. 71, 402–423.
Petri, J.L., Herter, F., 2002. Nashi pear (*Pyrus pyrifolia*) dormancy under mild temperate climate conditions. Acta Hortic. (ISHS) 587, 353–361.
Porto, D.D., Bruneau, M., Perini, P., Anzanello, R., Renou, J.P., dos Santos, H.P., Fialho, F.B., Revers, L.F., 2015. Transcription profiling of the chilling requirement for bud break in apples: a putative role for FLC-like genes. J. Exp. Bot. 66, 2659–2672.
Redestig, H., Fukushima, A., Stenlund, H., Moritz, T., Arita, M., Saito, K., Kusano, M., 2009. Compensation for systematic cross-contribution improves normalization of mass spectrometry based metabolomics data. Anal. Chem. 81, 7974–7980.
Rohde, A., Bhalerao, R.P., 2007. Plant dormancy in the perennial context. Trends Plant Sci. 12, 217–223.
Salvemini, F., Franzé, A., Iervolino, A., Filosa, S., Salzano, S., Ursini, M.V., 1999. Enhanced glutathione levels and oxidoresistance mediated by increased glucose-6-phosphate dehydrogenase expression. J. Biol. Chem. 274, 2750–2757.
Sansone, S., Fan, T., Goodacre, R., Griffin, J.L., Hardy, N.W., Kaddurah-Daouk, R., Kristal, B.S., Lindon, J., Mendes, P., Morrison, N., Nikolau, B., Robertson, D., Sumner, L.W., Taylor, C., van der Werf, M., van Ommen, B., Fiehn, O., 2007. The metabolomics standards initiative. Nat. Biotechnol. 25, 846–848.
Saure, M., 1985. Dormancy release in deciduous fruit trees. Hortic. Rev. 7, 239–300.
Seif El-Yazal, M.A., Seif El-Yazal, S.A., Rady, M.M., 2014. Exogenous dormancy-breaking substances positively change endogenous phytohormones and amino acids during dormancy release in 'Anna' apple trees. Plant Growth Regul. 72, 211–220.
Seif El-Yazal, M.A., Rady, M.M., 2012. Changes in nitrogen and polyamines during breaking bud dormancy in 'Anna' apple trees with foliar application of some compounds. Sci. Hortic. 136, 75–80.
Sherson, S.M., Alford, H.L., Forbes, S.M., Wallace, G., Smith, S.M., 2003. Roles of cell wall invertases and monosaccharide transporters in the growth and development of *Arabidopsis*. J. Exp. Bot. 54, 525–531.
Sugiura, T., Honjo, H., 1997. The effects of temperature on endodormancy completion in Japanese pear (*Pyrus pyrifolia* Nakai) and modeling the relationship. J. Agric. Meteorol. 53, 285–290.
Sugiura, T., Kuroda, H., Sugiura, H., 2007. Influence of the current state of global warming on fruit tree growth in Japan. J. Jpn. Soc. Hortic. Sci. 6, 257–263 (in Japanese with English abstract).
Taji, T., Ohsumi, C., Iuchi, S., Seki, M., Kasuga, M., Kobayashi, M., Yama guchi-Shinozaki, K., Shinozaki, K., 2002. Important roles of drought- and cold-inducible genes for galactinol synthase in stress tolerance in *Arabidopsis thaliana*. Plant J. 29, 417–426.
Takemura, Y., Kuroki, K., Matsumoto, K., Tamura, F., 2013. Cultivar and areal differences in the breaking period of bud endodormancy in pear plants. Sci. Hortic. 154, 20–24.
Takemura, Y., Kuroki, K., Shida, Y., Araki, S., Takeuchi, Y., Tanaka, K., Ishige, T., Yajima, S., Tamura, F., 2015. Comparative transcriptome analysis of the less-dormant taiwanese pear and the dormant Japanese pear during winter season. PLoS One 10http://dx.doi.org/10.1371/journal.pone.0139595. e0139595.
Tan, Y., Gao, D.S., Li, L., Chen, X.D., Xu, A.H., 2010. Respiratory response of dormant nectarine floral buds on chilling deficiency. Agric. Sci. in China 9, 1448–1454.
Victor, K.J., Fennell, A.Y., Grimplet, J., 2010. Proteomic analysis of shoot tissue during photoperiod induced growth cessation in *V. riparia* Michx. grapevines. Proteome Sci. 8, 44.
Viti, R., Andreini, L., Ruiz, D., Egea, J., Bartolini, S., Iacona, C., Campoy, J.A., 2010. Effect of climatic conditions on overcoming of dormancy in apricot flower buds in two Mediterranean areas: Murcia (Spain) and Tuscany (Italy). Sci. Hortic. 124, 217–224.
Wang, S.Y., Jiao, H.J., Faust, M., 1991. Changes in the activities of catalase, peroxidase, and polyphenol oxidase in apple buds during bud break induced by thidiazuron. J. Plant Growth Regul. 10, 33–39.
Wang, S.Y., Ji, Z.L., Faust, M., 1987. Metabolic changes associated with bud break induced by thidiazuron. J. Plant Growth Regul. 6, 85–95.
Wang, S.Y., Faust, M., 1988. Changes of fatty acids and sterols in apple buds during bud break induced by a plant bioregulator, thidiazuron. Physiol. Plantarum 72, 115–120.
Wang, S.Y., Faust, M., 1994. Changes in polyamine content during dormancy in flower buds of 'Anna' apple. J. Am. Soc. Hortic. Sci. 119, 70–73.
Weinberger, J.H., 1950. Chilling requirements of peach varieties. Proc. Am. Soc. Hortic. Sci. 56, 122–128.
Yoshioka, H., Nagai, K., Aoba, K., Fukumoto, M., 1988. Seasonal changes of carbohydrates metabolism in apple trees. Sci. Hortic. 36, 219–227.
Yu, L., Lan, W., Chao, C., Ying, Y., 2004. Glutathione levels control glucose-6-phosphate dehydrogenase activity during elicitor induced oxidative stress in cell suspension cultures of *Taxus chinensis*. Plant Sci. 167, 329–335.
Zanol, G.C., Sekozawa, Y., Sugaya, S., Gemma, H., 2010. The glutathione accumulation on mixed bud tissues during the dormancy of Japanese pear. Acta Hort. (ISHS) 872, 77–84.
Zhuang, W., Gao, Z., Wen, L., Huo, X., Cai, B., Zhang, Z., 2015. Metabolic changes upon flower bud break in Japanese apricot are enhanced by exogenous GA_4. Hortic. Res. 2, 15046.
Zuther, E., Büchel, K., Hundertmark, M., Stitt, M., Hincha, D.K., Heyer, A.G., 2004. The role of raffinose in the cold acclimation of *Arabidopsis thaliana*. FEBS Lett. 576, 169–173.